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Title: Investigating differences in the ability of XplA/B-containing bacteria to degrade the explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)

Authors: Dana Khdr Sabir[1,2], Nicolas Grosjean[1], Elizabeth L. Rylott[1], Neil C. Bruce[1]*

[1]Centre for Novel Agricultural Products, Department of Biology, University of York, Wentworth Way, York, YO10 5DD, UK

[2] Department of General Sciences, Charmo University, 46023 Chamchamal, Sulaimani, Kurdistan Region- IRAQ

*Correspondence to: Neil C. Bruce, Centre for Novel Agricultural Products, Department of Biology, University of York, Wentworth Way, York, YO10 5DD, UK

Phone: +44 (0)1904 328777

Fax: +44 (0)1904 328801

E-mail: neil.bruce@york.ac.uk

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Footnote: NG now at Laboratoire Interdisciplinaire des Environnements Continentaux, UMR 7360 CNRS - Université De Lorraine, 54506 Vandoeuvre-Les-Nancy, France

Graphical Abstract and One-Sentence Summary

Differences in the ability of three bacterial strains to degrade RDX, an explosive and environmental pollutant, were investigated using sequence and biochemical analyses.

Abstract

The xenobiotic hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a toxic explosive and environmental pollutant. This study examines three bacterial species that degrade RDX, using it as a sole source of nitrogen for growth. Although isolated from diverse geographical locations, the species contain near identical copies of genes encoding the RDX-metabolizing cytochrome P450, XplA, and accompanying reductase, XplB. Sequence analysis indicates a single evolutionary origin for *xplA* and *xplB* as part of a genomic island, which has been distributed around the world via horizontal gene transfer. Despite the fact that *xplA* and *xplB* are highly conserved between species, *Gordonia* sp. KTR9 and *Microbacterium* sp. MA1 degrade RDX more slowly than *Rhodococcus rhodochrous* 11Y. Both *Gordonia* sp. KTR9 and *Microbacterium* sp. MA1 were found to contain single base pair mutations in *xplB* which, following expression and purification, were found to encode inactive XplB protein. Additionally, the *Gordonia* sp. KTR9 XplB was fused to glutamine synthetase, which would be likely to sterically inhibit XplB activity. Although the glutamine synthetase is fused to XplB and truncated by 71 residues, it was found to be active. Glutamine synthetase has been implicated in the regulation of nitrogen levels; controlling nitrogen availability will be important for effective bioremediation of RDX.

Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a toxic explosive widely used in military munitions. The use of RDX on military lands, coupled with its recalcitrance to biodegradation, has resulted in the build-up of significant levels of pollution. This synthetic nitramine, which has no known equivalent structure in nature, has been in the environment for less than a century but nevertheless, microorganisms have evolved the ability to degrade it (Rylott *et al.*, 2011). The enzymes involved in the aerobic biodegradation of RDX were first

isolated in the UK from *Rhodococcus rhodochrous* 11Y and identified as an unusual cytochrome P450, XplA, and accompanying flavodoxin reductase partner, XplB (Seth-Smith *et al.*, 2002). A number of aerobic RDX-degrading bacteria have been reported (Binks *et al.*, 1995, Coleman, 1998, Thompson *et al.*, 2005, Indest *et al.* 2007, Nejidat *et al.* 2008, Andeer *et al.*, 2009, Bernstein *et al.*, 2011,) from different geographical locations but all belonging to the order Actinomycetales.

Within all the species tested, *xplA* has been detected, and found to have greater than 99 % identity (Indest *et al.*, 2007, Seth-Smith *et al.*, 2008, Andeer *et al.*, 2009, Bernstein *et al.*, 2011, Rylott *et al.*, 2011, Chong *et al.*, 2014). The highly conserved nature of *xplA* suggests its rapid distribution by horizontal gene transfer (Seth-Smith *et al.*, 2008, Andeer *et al.*, 2009, Rylott *et al.*, 2011, Chong *et al.*, 2014). Genes involved in xenobiotic catabolism are often located on a mobile genetic element, accompanied by insertion elements, and are integrated into the bacterial chromosomal or plasmid (Nojiri *et al.*, 2004). In agreement with this, *xplA* is plasmid-encoded in *Microbacterium* sp. MA1, *R. rhodochrous* 11Y (Andeer *et al.*, 2009) and *Gordonia* sp. KTR9 (Indest *et al.*, 2010). Partial sequence analysis of the plasmid carrying *xplA* in *Microbacterium* sp. MA1 revealed that the gene is also associated with transposable elements in this bacterium (Andeer *et al.*, 2009). Furthermore, a 6.7 kbp region flanking *xplA* has been found that is nearly identical (> 99 %) between *Microbacterium* sp. MA1 and *R. rhodochrous* 11Y (Andeer *et al.*, 2009).

The partnering reductase for XplA is XplB (Seth-Smith *et al.*, 2002), an NADPH-dependent flavoprotein which contains one molecule of FAD as a cofactor and shares sequence homology (and 27 % sequence identity) with the bovine adrenodoxin reductase FDXR (US National Library of Medicine National Institutes of Health (NCBI) accession number P08165.3). The reductase XplB is involved in the activation of the catalytic centre of XplA

via the transfer of electrons from NADPH to a flavodoxin domain fused to the N-terminal of the P450 domain of XplA (Jackson *et al.*, 2007).

The role of XplB in RDX degradation has been demonstrated in both *R. rhodochrous* 11Y (Chong *et al.*, 2014) and transgenic plant lines (Jackson *et al.*, 2007, Bui *et al.*, 2012). In *Gordonia* sp. KTR9 the 5′ end of *xplB* is fused to a glutamine synthetase (GS)-encoding gene, *glnA* (Indest *et al.*, 2010), an arrangement that has not been found in any of the other RDX-degrading bacteria examined so far. Glutamine synthetase (EC 6.3.1.2) is an essential enzyme in nitrogen metabolism, catalysing the ATP-dependant production of glutamine from glutamate and ammonia.

Previously, we reported that although *xplA* and *xplB* are highly conserved amongst RDX-degrading bacteria, the ability of *Gordonia* sp. KTR9 and *Microbacterium* sp. MA1 to grow in minimal medium with RDX as the sole source of nitrogen was significantly less than that of *R. rhodochrous* 11Y. The ability of these bacteria to remove RDX from the medium correlated with growth rates (Chong *et al.*, 2014). It is possible that the fusion of *xplB* with *glnA* and/or additional changes in the genetic components and arrangements of the genes in this region may account for differences in the regulation of RDX degradation in *Gordonia* sp. KTR9 (Zhu *et al.*, 2014).

Here we present analysis of the *xplA* and *xplB*-containing gene clusters from *R. rhodochrous* 11Y, *Microbacterium* sp. MA1 and *Gordonia* sp. KTR9, which reveal differences and commonalities in the arrangement of genes; along with emphasis on the sequence of *xplB*, and characterisation of the GS-XplB fusion from *Gordonia* sp. KTR9.

Materials and Methods

Genome sequencing

Whole genome sequencing was used to obtain the complete *xplA*/*B* genomic island of *R. rhodochrous* 11Y (NCBI acc. no. KY488543; Figure 1). To do this, total genomic DNA was extracted by lysing the cells in TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0) containing 10 mg/ml lysozyme, 10 % SDS and 20 mg/ml proteinase K. Cell debris was centrifuged at 10000rpm and protein removed by phenol-chloroform extraction. Nucleic acids were precipitated with 3M sodium acetate and isopropanol, washed in 70 % ethanol and resuspended in nuclease-free water. Prior to sequencing, the 16S rRNA region was sequenced to ensure that the DNA sample was from the correct species. Additionally, the *xplA* gene was amplified from the DNA sample to make sure that the plasmid had not been cured from the bacterial genome during the extraction process. The DNA sample was analysed using an Agilent TapeStation 2200 and sequenced using a Next Generation Sequencing platform Ion Torrent (Life Technologies). Raw sequence reads were assembled using Newbler, version 2.7 (Roche Diagnostics).

Construction of mutant strains

Point mutations in *xplB* and *glnA-xplB* were obtained using the QuikChangeII Site-Directed Mutagenesis protocol (Agilent Technologies Inc.). To mutate Trp-386 to Ser in *R. rhodochrous* 11Y XplB, primers *xplB* (W386S)-F 5’ GTC GAT TTC GAC GGC TCG ATG CGG ATC G-3’ and *xplB* (W386S)-R 5’- CGT CGA TCC GCA TCG AGC CGT CGA AA-3’ were used. To mutate the Ser-385 to Trp in GS-XplB, primers *xplB* (S385W)-GS-F 5’-GTC GAT TTC GAC GGC TGG ATG CGG ATC G-3’ and *xplB* (S385W)-GS-R 5’-CGT CGA TCC GCA TCC AGC CGT CGA AA-3’ were used. To mutate Phe-172 to Ile in *R. rhododochrous* 11Y XplB, primers *xplB* (F172I)-F 5’ AAG CAG CCC GAC GAA ATC ACC GGT TC-3’ and *xplB* (F172I)-R 5’ ATC GGA ACC GGT GAT TTC GTC GGG C-3’ were used. Mutations were confirmed by sequencing.

Cloning and expression

The *glnA-xplB* gene was amplified from *Gordonia* sp. KTR9 by PCR using primers pGEX-*xplB-glnA*-F 5'- GGTTCCGCGTGGATCCATGAGTACATCCGCGCTCG-3' and pGEX-x*plB-glnA*-R 5'- GTCGACCCGGGAATTCTCAGCAGACCGATTCGGCCG-3' and cloned at the *BamH*I and *EcoR*I restriction sites, using an In-Fusion® HD cloning system, into pGEX2T. The GS-XplB fusion protein was expressed in *Escherichia coli* BL-21 (DE3). The cells were grown at 37 ºC to OD600 ~0.6, then induced with 0.5 mM IPTG supplemented with 50 µg/ml riboflavin and grown for 14 hours at 20 ºC. All proteins were expressed and purified as described by Jackson *et al.* (2007), protein identities were confirmed by MALDI-MS sequence analysis.

Activity assays

Reductase activity of XplB homologs was determined using 75 µl of cell free extract or 100 µg purified protein, 50 mM potassium phosphate buffer (pH 6.8), 300 µM NADPH, 0.08 mg purified XplA protein (Jackson *et al.*, 2007) (0.65 mg for GS-XplB assays) and 100 µM RDX, in a final volume of 1 mL. For this assay, *R. rhodochrous* 11Y XplA was first purified (Figure 2a), and its activity towards RDX verified using spinach ferredoxin reductase (Sigma-Aldrich), as reported previously (Rylott *et al.*, 2006). The reactions were initiated by the addition of 100 µM of RDX at room temperature and time points samples stopped by the addition of 10 % (v/v) 1M trichloroacetic acid. Levels of RDX were measured using HPLC (Jackson *et al.*, 2007), and nitrite production using Griess reagent (Griess, 1879) as follows: To 180 µl of sample, 50 µL of 10 mg of sulfanilamide/ml in 0.68 M HCl was added and incubated for 5 minutes, and then 20 µL of 10 mg of *N*-(1-naphthyl)-ethylenediamine dihydrochloride in water were added. Following mixing, and a further 10 min incubation at room temperature, absorbance at 540 nm was measured. Sodium nitrite (0 to 100 µM nitrite)

was used as a standard. Glutamine synthetase was measured spectrophotometrically, based on the method of Kingdon *et al.*, 1968 and using a pyruvate kinase and lactic dehydrogenase-linked assay that followed reduction of NADH at wavelength 340nm. Each 3 mL reaction mix cuvette contained 34.1 mM imidazole buffer pH 7.1, 102 mM sodium glutamate, 8.5 mM adenosine 5'-triphosphate, 1.1 mM phosphoenolpyruvate, 60 mM magnesium chloride, 18.9 mM potassium chloride, 45 mM ammonium chloride, 0.25 mM b-nicotinamide adenine dinucleotide, 28 units pyruvate kinase, 40 units L-lactic dehydrogenase and 0.4 - 0.8 unit glutamine synthetase. One enzyme unit will convert 1.0 µmole of L-glutamate to L-glutamine in 1 minute at pH 7.1 at 37°C with specific activity defined as number of units per mg protein.

Measurement of FAD

The amount of FAD cofactor bound to the XplB and GS-XplB proteins was measured following the method described by Aliverti *et al.*, 1999. Protein was boiled at 100 ºC, in the dark, for 20 min and precipitated protein removed by centrifugation at 13,000 rpm for 10 minutes. The UV-visible spectrum of the supernatant was recorded (200 to 600 nm) spectrophotometrically. FAD was also determined by HPLC analysis using a C18 column with a mobile phase of 5 mM ammonium acetate buffer, pH 6.5 (solvent A) and methanol (solvent B) and the following gradient: 5 minutes solvent A 85 %: solvent B 15 %; 20 minutes solvent A 25 %: solvent B 75 %; 5 minute solvent A 0 %: solvent B 100 %; 5 minutes solvent A 85 %: solvent B 15 %.). The flow rate was 0.75 ml/min and column temperature 30 ºC. Commercially available FAD (Sigma) was used as a reference.

Results

The *xplA*/*B* gene clusters

Analysis of the putative *xplA*/*B* genomic islands in *R. rhodochrous* 11Y, *Microbacterium* sp. MA1 and *Gordonia* sp. KTR9 is shown in Figure 1. Within a 53 kb region in *R. rhodochrous* 11Y, there are 13.8 kb and 11.8kb gene clusters (termed the A and B regions respectively). Further downstream, is a 570 bp sequence encoding a transposable element (termed the C region). While the A region, which contains *xplA* and *xplB*, is highly conserved between *R. rhodochrous* 11Y and *Microbacterium* sp. MA1, *Gordonia* sp. KTR9 shares homology only with 3.1 kbp of the A region, although this includes *xplA* and *xplB*. Conversely, the B and C regions are highly conserved between *R. rhodochrous* 11Y and *Gordonia* sp. KTR9, but absent from *Microbacterium* sp. MA1. Two additional regions, (termed D1 and D2) are nearly identical between *Microbacterium* sp. MA1 and *Gordonia* sp. KTR9; yet absent from *R. rhodochrous* 11Y. Scattered within these gene clusters are a number of transposable elements, indicative of mobility within and between these regions.

Microbacterium sp. MA1 XplB

The only difference between the *R. rhodochrous* 11Y and *Microbacterium* sp. MA1 XplB sequences is that *R. rhodochrous* 11Y contains a Phe-172, which is conserved across a range of RDX-degrading bacterial genera, whereas *Microbacterium* sp. MA1 contains an Ile-172 (Table 1). In *R. rhodochrous* 11Y XplB, a protein-bound flavin (FAD) has been reported to be loosely bound (Jackson *et al.*, 2007). In agreement with this, purified *R. rhodochrous* 11Y XplB contained 25 % of the predicted FAD and was yellow-colored. However, the *Microbacterium* sp. MA1 XplB protein was colorless and lacked detectable FAD (Figure 2b and c). Reductase activity in cell lysates was measured using the Griess assay to detect nitrite released during the degradation of RDX by XplA. Figure 2d shows that in reaction mixtures containing XplA and lysate from cells expressing *R. rhodochrous* 11Y XplB, nitrite release was observed. However, although nitrite was detected in reaction mixtures containing lysate

from cells expressing the *Microbacterium* sp. MA1 XplB, the level was not significantly different from that seen from lysate transformed with the empty vector control, indicating that *Microbacterium* sp. MA1 XplB was inactive.

Characterisation of the XplB portion of the *Gordonia* KTR9 GS-XplB fusion

In *Gordonia* KTR9, the sequence of the XplB portion of the GS-XplB fusion was found to differ from the *R. rhodochrous* 11Y XplB sequence by just two amino acids. Firstly, the Met-1 in *Gordonia* KTR9 GS-XplB was missing, enabling the uninterrupted translation of XplB following GS to produce the GS-XplB fusion. Secondly, *R. rhodochrous* 11Y XplB contained a tryptophan (Trp-386) whereas the *Gordonia* KTR9 GS-XplB fusion contained serine (Ser-385). This difference was the result of a single base change: TCG encoding tryptophan and TGG encoding serine. A multiple sequence alignment, by % identity, of XplB revealed that Trp-386 is highly conserved across a number of bacterial genera (Table 1). Purified *Gordonia* KTR9 GS-XplB fusion protein was colorless and lacked detectable FAD (Figure 2b and c). Subsequent Griess assays suggested that the GS-XplB fusion was inactive (Figure 2d) and to confirm this, RDX removal rates by cell lysates were measured. When lysate from cells expressing *R. rhodochrous* 11Y XplB was used as the source of reductase, all the RDX was removed within 10 minutes. Although RDX degradation was detected in reaction mixtures containing lysate from cells expressing the *Gordonia* KTR9 GS-XplB fusion, this was lower than endogenous *E. coli* reductase activity measured in lysate from cells transformed with just the empty vector control (Figure 2e). Together, these results demonstrate that the XplB component of the *Gordonia* KTR9 GS-XplB was inactive.

To investigate further, the role of Ser-385 and Trp-386 in XplB activity, reciprocal mutations in *R. rhodochrous* 11Y XplB (W386S) and the *Gordonia* KTR9 GS-XplB-S385W fusion were made. Lysate from cells expressing *R. rhodochrous* 11Y XplB-W386S lacked the

yellow coloration observed in lysate from unmutated *R. rhodochrous* 11Y XplB. Furthermore, when used as the partnering reductase for XplA, activity towards RDX was only observed when unmutated *R. rhodochrous* 11Y XplB was supplied as the reductase; no activity was observed in assays using the mutated *R. rhodochrous* 11Y XplB-W386S (Figure 3a). Whereas lysate from cells expressing the *Gordonia* KTR9 GS-XplB fusion was not yellow colored, lysate from cells expressing the mutated *Gordonia* KTR9 GS-XplB-S385W fusion had the same yellow coloration observed in the cell lysate of *R. rhodochrous* 11Y XplB. Figure 3b shows that when mutated *Gordonia* KTR9 GS-XplB-S385W fusion lysate was supplied as a reductase in Griess assays, with purified *R. rhodochrous* 11Y XplA and RDX as substrate, nitrite was detected. In assays measuring RDX using HPLC, lysate from the mutated *Gordonia* KTR9 GS-XplB-S385W fusion removed significantly more RDX than lysate from cells transformed with the empty vector (Figure 3c). However, upon purification, the mutated *Gordonia* KTR9 GS-XplB-S385W fusion protein appeared colorless and FAD was not detectable using HPLC. Assays monitoring the production of nitrite from RDX by *R. rhodochrous* 11Y XplA, confirmed that the purified, mutated *Gordonia* KTR9 GS-XplB-S385W fusion was inactive.

Characterization of the GS portion of the *Gordonia* KTR9 GS-XplB fusion

Multiple sequence alignments with characterized GS type I protein sequences (Brown *et al.*, 1994, Hayward *et al.*, 2009, Murray *et al.*, 2013) revealed that the GS component of the *Gordonia* KTR9 GS-XplB fusion belongs to the GS type I-α class, which lack the insertion signature sequence found in the GS type I-β class (Brown *et al.*, 1994; Table 2). The *Gordonia* KTR9 GS-XplB contains 390 amino acids and alignment with the three closest homologs (> 78 % identity) available in public databases revealed that the fusion protein is truncated, missing 71 amino acids from the C-terminus. To investigate whether the missing

residues form part of the active site, a model structure of the GS portion was created based on the closest homolog (29.1 % identity) in the protein database bank for which structural data was available: GS from *B. subtilis* (acc. no. P12425). Superimposing the model structure of *Gordonia* KTR9 GS-XplB on the GS from *B. subtilis* (RMSD: 1.141 Å) revealed the structure, and position, of the missing region (Figure 4a). Based on this homology modelling, and knowledge from the structure of the GS type I-α of *B. subtilis* (Murray *et al.*, 2013), it was found that the missing residues in the GS fusion included two residues involved in the formation of a Tyr loop in the active site. Residues located on the other loops mediating the catalytic activity of the enzyme present in GS from *B. subtilis* were also found in the *Gordonia* KTR9 GS-XplB. This included an Asn loop which shares remarkable similarity with GS from *B. subtilis*, while the Asp^{50}, on a latch loop was found to be extended in the *Gordonia* KTR9 GS-XplB when compared to the GS from *B. subtilis*. Remarkably, despite fusion and truncation, the GS-XplB protein has glutamine synthetase activity (Figure 4b).

Discussion

Both *xplA* and *xplB* are highly conserved amongst different genera of aerobic RDX-degrading bacteria isolated from distinct geographical locations. This conservation endorses the theory of the recent evolution of these genes, and dissemination around the world through horizontal gene transfer (Seth-Smith *et al.*, 2008, Andeer *et al.*, 2009, Jung *et al.*, 2011). Such evolution and distribution has been reported for other xenobiotic-degrading genes, for example naphthalene degrading genes (*nahAc*) (Herrick *et al.*, 1997), N-heterocycle morpholine degrading genes (*morABC*) (Sielaff & Andreesen, 2005) and atrazine degrading genes (*atzABC*) (de Souza *et al.*, 1998) were also found to be highly conserved to the level of identical copies of the same gene found amongst different bacteria isolated from diverse geographical locations.

In addition to *xplA* and *xplB*, there are several neighboring genes which are nearly identical between the bacteria studied here. In *R. rhodochrous* 11Y, *Microbacterium* sp. MA1 (Andeer *et al.*, 2009) and *Gordonia* sp. KTR9 (Indest *et al.*, 2010), there are transposable elements in neighboring regions, suggesting that *xplA* and *xplB* are part of a larger mobile element such as an Integrative and Conjugative Element (ICE) or genomic island in a conjugative plasmid. Similarly, the genes for the degradation of xenobiotics such as chlorobenzoate, by *Pseudomonas* sp. strain B13 (Ravatn *et al.*, 1998, Gaillard *et al.*, 2006); and biphenyl degradation by *Ralstonia eutropha* A5 (Springael *et al.*, 2001), are also found partly on larger mobile elements. Genomic islands and ICEs are known to excise and integrate into chromosomes or plasmids through conjugation (Burrus *et al.*, 2002, van der Meer & Sentchilo, 2003). In support of this, *R. rhodochrous* 11Y.058 (Figure 1) encodes a phage related integrase belonging to the tyrosine recombinase family, which shares complete identity with a site-specific recombinase from *R. erythropolis* PR4. Moreover, the RDX-degradation capacity of *Gordonia* sp. KTR9 was successfully transferred into the non-RDX degrading species *Gordonia polyisoprenivorans*, *Rhodococcus jostii* RHA1 and *Nocardia* sp. TW2 through conjugation (Jung *et al.*, 2011), demonstrating that this is a possible transfer mechanism.

Sequence analysis of the genes surrounding *xplA* and *xplB* showed that there are regions of identity between the three species, and although *xplA* alone is sufficient for the catabolism of RDX (Rylott *et al.*, 2006, Indest *et al.*, 2010), it has been speculated that some of these neighboring genes contribute towards RDX degradation (Indest *et al.*, 2010, Indest *et al.*, 2013, Chong *et al.*, 2014, Zhu *et al.*, 2014). For example, downstream of *xplB* in *R. rhodochrous* 11Y and *Microbacterium* sp. MA1 are genes encoding a putative permease (AroP; 11Y.026 and MA1.029 in Figure 1) and transcriptional regulator (MarR; 11Y.025 and

MA1.027 in Figure 1). Orthologous permeases have been found close to genes with related function (Wehrmann *et al.*, 1995, Yu *et al.*, 2007), and members of the MarR family shown to play a role in regulating catabolism of aromatic compounds (Bussmann *et al.*, 2010; Chong *et al.*, 2014). However, gene deletion analysis has demonstrated that AroP and MarR from *R. rhodochrous* 11Y do not affect RDX degradation in this species (Chong *et al.*, 2014). In *Gordonia* sp. KTR9, sequence analysis of pGKT2, the 182 kb plasmid carrying *xplA* and *xplB* indicated that they are integrated into an operon involved in the degradation of N-heterocyclic compounds (Zhu *et al.*, 2014). On this operon, the genes upstream of *xplA* and *xplB* (*xplR*, *cyp151C* and *glnA*) share high sequence similarity and arrangement to the *mor* and *pip* gene clusters involved in the degradation of morpholine, piperidine and related compounds in *Mycobacterium* and *Rhodococcus* (Indest *et al.*, 2010).

An *xplB* knock-out in *R. rhodochrous* 11Y demonstrated that although XplB is not required for XplA activity; the absence of XplB reduces the rate of RDX-degradation by 70 % (Chong *et al.*, 2014). The fact that an *xplB* knock-out can still degrade RDX indicates that alternative endogenous reductases can substitute in bacteria, and this has also been demonstrated in XplA-transformed plants (Jackson *et al.*, 2007). A previous comparison between the RDX removal rates of RDX-degrading isolates showed that all *Rhodococcus* spp. had faster RDX removal rates than *Microbacterium* sp. MA1 or *Gordonia* sp. KTR9 (Chong *et al.*, 2014). Considering that XplA alone is able to denitrate the RDX structure in the organism (Rylott *et al.*, 2006, Indest *et al.*, 2010), and is identical in species across all three genera (Chong *et al.*, 2014), the differences in the RDX-removal rate are due to the impairment of XplB, differences in bacterial physiology, or a combination of both.

The studies presented here indicate that mutations present in XplB in *Gordonia* sp. KTR9 and *Microbacterium* sp. MA1 explain, to a degree, why these species exhibit reduced rates of

RDX degradation when compared with *R. rhodochrous* 11Y. Our studies comparing the *R. rhodochrous* 11Y Trp-386 with the Ser-385 found in *Gordonia* sp. KTR9 showed that Trp-386 clearly plays a critical role in retaining FAD in XplB. This residue also appears to be important for the functionality of other FAD containing proteins as multiple sequence alignments revealed the residue to be highly conserved amongst FAD-containing proteins that share as low as 42 % sequence identity with XplB. Additionally, in *Gordonia* sp. KTR9, the fusion of GS to XplB is likely to further inhibit the reductase activity of XplB, and thus RDX-degrading activity of *Gordonia* sp. KTR9. Considering that *Gordonia* sp. KTR9 was isolated from soil where RDX was not detected (Thompson *et al.*, 2005), it is possible that the recombination and reduction of the genomic island, which resulted in the fusion of the XplB to GS, arose from the absence of selective pressure from RDX.

Despite the fact that the GS component of the *Gordonia* sp. KTR9 GS-XplB fusion is truncated, it was found to be active, demonstrating that the purified GS protein is correctly folded and the missing residues are not essential for functionality. The GS portion of the *Gordonia* sp. KTR9 GS-XplB fusion belongs to GS type I-α. It is already known that the GS type I-α from *B. subtilis* plays an important role in regulating cellular nitrogen levels by controlling the expression of GlnR and TnrA. In the presence of glutamine, GS binds TnrA directly onto the DNA, forming a GS-TnrA-DNA complex with its formation regulated by the intracellular levels of ATP, AMP, glutamine, and glutamate. (Fisher & Wray, 2008, Wray & Fisher, 2010, Murray *et al.*, 2013, Hauf et al., 2016). Mutation of GS in *B. subtilis* resulted in the constitutive expression of both GlnR and TnrA proteins (Wray & Fisher, 2010). In *Gordonia* sp. KTR9, GlnR is important in the assimilation of nitrite in the cell. Knock-out of *glnR* in *Gordonia* sp. KTR9 resulted in the accumulation of nitrite from RDX (Zhu *et al.*, 2014) and down-regulation of the nitrite reductase gene loci KTR9_1306 and KTR9_1307

(Indest *et al.*, 2013). Additionally, the *glnR* mutant lacked the ability to utilise RDX, nitrite or nitrate as the sole source of nitrogen, but not ammonium or glutamine.

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Figure Legends

Figure 1. Schematic representation of the RDX-degrading gene clusters in three genera of aerobic RDX-degrading bacteria.

White colored regions have no sequence homology. Dotted lines indicate unsequenced regions, genes encoding transposable elements are shown in yellow.

Figure 2. Purification and analysis of XplB proteins from *R. rhodochrous* 11Y, *Microbacterium* sp. MA1 and *Gordonia* sp. KTR9.

(**a**) SDS-PAGE analysis of lysates (L) and purified proteins from *E. coli* cells expressing XplA or XplB homologues. (**b**) Overlaid chromatograms showing FAD absorbance peaks from purified proteins. (**c**) Appearance of purified proteins. (**d**) Reductase activity in cell lysates expressing XplB homologues, measured using the Griess assay with purified XplA and RDX as substrate (n = 3 ± SD. Letters refer to significant differences; ANOVA, Tukey HSD). (**e**) RDX removal from *E. coli* cell lysates expressing *R. rhodochrous* 11Y XplB and *Gordonia* sp. KTR9 GS-XplB supplemented with purified XplA (n = 3 ± SD).

Figure 3 Activities of XplB proteins carrying reciprocal mutations from *R. rhodochrous* 11Y and *Gordonia* sp. KTR9.

(**a**) Nitrite release from *E. coli* cell lysates expressing *R. rhodochrous* 11Y XplB and *R. rhodochrous* 11Y XplB-(W386S) measured using the Griess assay, with purified XplA and RDX as substrate (n = 3 ± SD). (**b**) Nitrite release, observed using the Griess assay, by *E. coli* cell lysates expressing *R. rhodochrous* 11Y XplB and *Gordonia* sp. KTR9 GS-XplB-(S385W), with purified XplA and RDX as substrate. (**c**) RDX removal by *E. coli* cell lysates expressing the *Gordonia* sp. KTR9 GS-XplB-(S385W) or empty vector (n = 3 ± SD).

Figure 4 Characterization of the glutamine synthetase (GS) and XplB portions of *Gordonia* sp. KTR9 GS-XplB fusion protein.

(**a**) Model structure of GS from GS-XplB (blue) superimposed on the GS structure of *B. subtilis* (yellow). Sequence missing from GS-XplB (red). Root mean square deviation 1.14 ºA). (**b**) GS activity in *E. coli* cell lysates (n = 3 ± SD).

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Table 1

Multiple sequence alignment of the *Rhodococcus rhodochrous* 11Y XplB showing closest matches by % identity. The alignment shows the regions containing the phenylalanine-172 (F) residue that is replaced with isoleucine (I) in *Microbacterium* sp. MA1; and the tryptophan-386 (W) residue that in the *Gordonia* sp. KTR9 GS-XplB fusion, corresponds to serine (S). Multiple species for genera have been omitted. The alignment was performed using ClustalW2 (EMBL European Bioinformatics Institute).

Organism	**Identity (%)**	**UniProt AC**	Sequence (N- to C-terminal)
Rhodococcus rhodochrous 11Y	100	Q8GPH8	…DIIRLLSKQPDE**F**TGSD…**W**MRIDEAEVASASPGRIRQKVREVD…
Gordonia sp. KTR9	99.8	E1R0R9	…DIIRLLSKQPDE**F**TGSD…**S**MRIDEAEVASASPGRIRQKVREVD…
Microbacterium sp. MA1	99.8	C3UMY2	…DIIRLLSKQPDE**I**TGSD…**W**MRIDEAEVASASPGRIRQKVREVD…
Pseudomonas sp.	47.7	A0A0V8SZM6	…DVLRLLAKSAED**F**AGSD…**W**QRIDHAETRAAAPGRVRRKITDRA…
Nitratireductor pacificus	47.2	K2MCG5	…DLLRLLAKTPDE**L**AGSD…**W**KRIDAAEIAAAPENRCRVKINSRD…
Marinovum algicola	46.7	A0A0H4L107	…DLLRLLAKSPEE**L**EGSD…**W**CRIDSAETANPPPGRCRAKITTRE…
Ventosimonas gracilis	44.5	A0A139SRD6	…DVLRLLIKPAHD**W**QGSD…**W**QRIDTEEKQCAPAGRVRQKITERV…
Roseomonas mucosa	44.5	A0A0W0A9P2	…DLARMLSKDEAE**L**AGSD…**W**LRIRAAEEAAASAGRVRRKGATRD…
Mameliella alba	44.4	A0A0B3RZQ4	…DLLRLLAKAPEE**L**DGSD…**W**SRIDAAETGAAPEGRCRTKLATRE…
Ruegeria sp.	44.1	A0A1E3D8F1	…DLLRLLAKAPEE**L**DGSD…**W**SRIDAAETGAAPEGRCRTKLATRE…
Agrococcus jejuensis	43	A0A1G8CDN7	…DVARLVARDAAD**F**DGTD…**W**RRIDAAETLAAAPGRRRAKLRTLD…
Agrococcus pavilionensis	43	U1LRN2	…DVVRLLARDAEG**L**GGTD…**W**RRIDVREQLGAAPGRSRSKLRSRA…
Arthrobacter globiformis	43	H0QK39	…DVVRFLIKTPGE**F**AGSD…**W**LRVDAAERAAAPGNRSRKKLPDHA…
Leucobacter sp.	42.1	A0A061LTW8	…DVLRFLVKDRDA**Y**EGSD…**W**LRLDEHERSVAPSGRVRHKLPDHD…

Table 2.

Multiple sequence alignment of a region of the glutamine synthetase component of the GS-XplB fusion from *Gordonia* sp. KTR9. The shaded region is unique to class GS type I-β type GS (Brown *et al.*, 1994). The alignment was performed using ClustalW2 (EMBL European Bioinformatics Institute**).**

Organism	**Class**	**UniProt AC**	Sequence (N- to C-terminal)	
Mycobacterium tuberculosis	GSI-β	P9WN39	STGIADTAYFGAEAEFYIFDSVSFDSRANGSFYEVDAISGWWNTGAATEA	170
Streptomyces coelicolor	GSI-β	P15106	STGIADTAFFGPEAEFYVFDSVRFATRENESFYHIDSEAGAWNTGALED-	165
Synechococcus sp.	GSI-β	P28605	ASGIGDTAYFGPEAEFFVFDDVRFDQTENKGFYYVDSVEGRWNSGRKEP-	168
Anabaena sp.	GSI-β	K7W630	STGLGDTAFFGPEAEFFIFDDVRYDQTTNSGYYYVDSVEGRWNTGREE--	166
Salmonella typhimurium	GSI-β	P0A1P6	ATGIADTVLFGPEPEFFLFDDIRFGASISGSHVAIDDIEGAWNSSTKYE-	166
Pyrococcus furiosus	GSI-α	Q05907	KE--GYKAYIGPEPEFYLFK----------KNGTWELEIPDV-	141
Haloferax volcanii	GSI-α	P43386	ELGY-DVNVA-PEPEFFLFEE----------DEDGRATTVTNDA-	164
Bacillus subtilis	GSI-α	P12425	DLGFSDFNLG-PEPEFFLFKL----------DEKGEPTLELNDK-	153
Methanococcus voltae	GSI-α	P21154	EEFKGEYFVG-PEPEFFILK----------NENGK--WVPGDD-	156
Gordonia sp. KTR9	GSI-α	E1R0R9	ERTGLEMRTG-TEPEMTWEG----------EGFETTFRPDS-	176

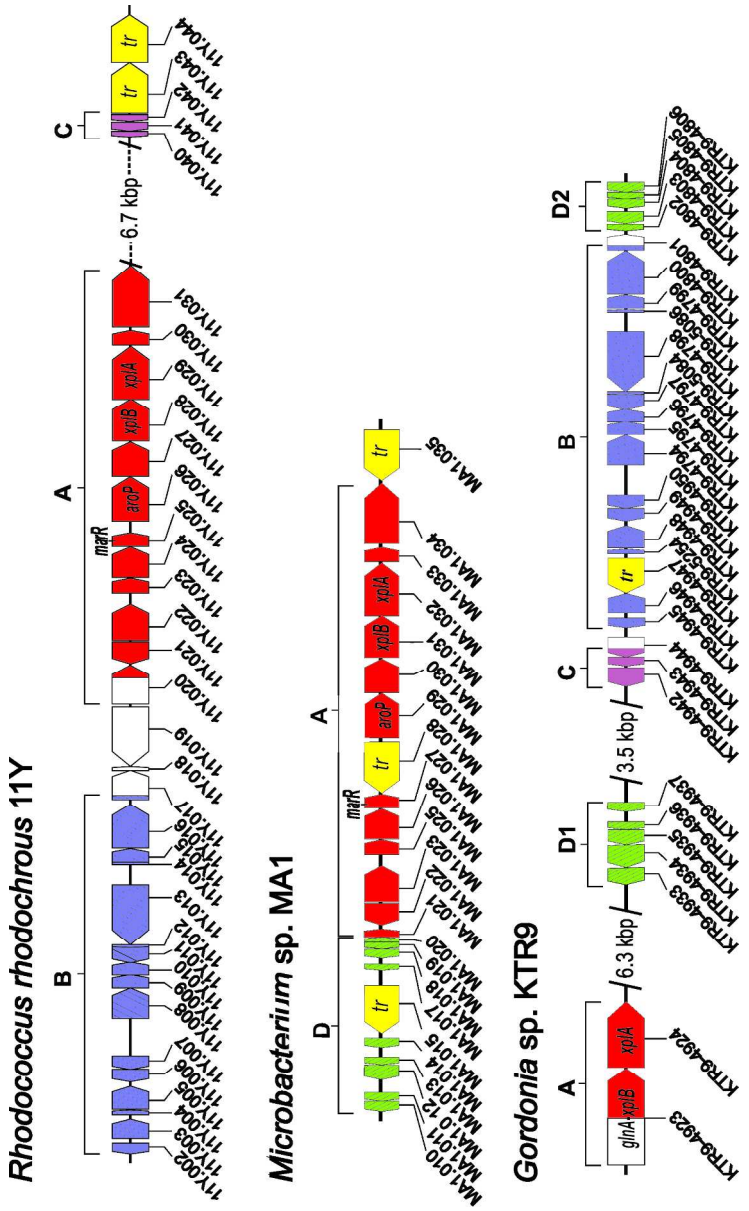

Figure 1. Schematic representation of the RDX-degrading gene clusters in three genera of aerobic RDX-degrading bacteria.
White colored regions have no sequence homology. Dotted lines indicate unsequenced regions, genes encoding transposable elements are shown in yellow.
Figure 2. Purification and analysis of XplB proteins from R. rhodochrous 11Y, Microbacterium sp. MA1 and Gordonia sp. KTR9.

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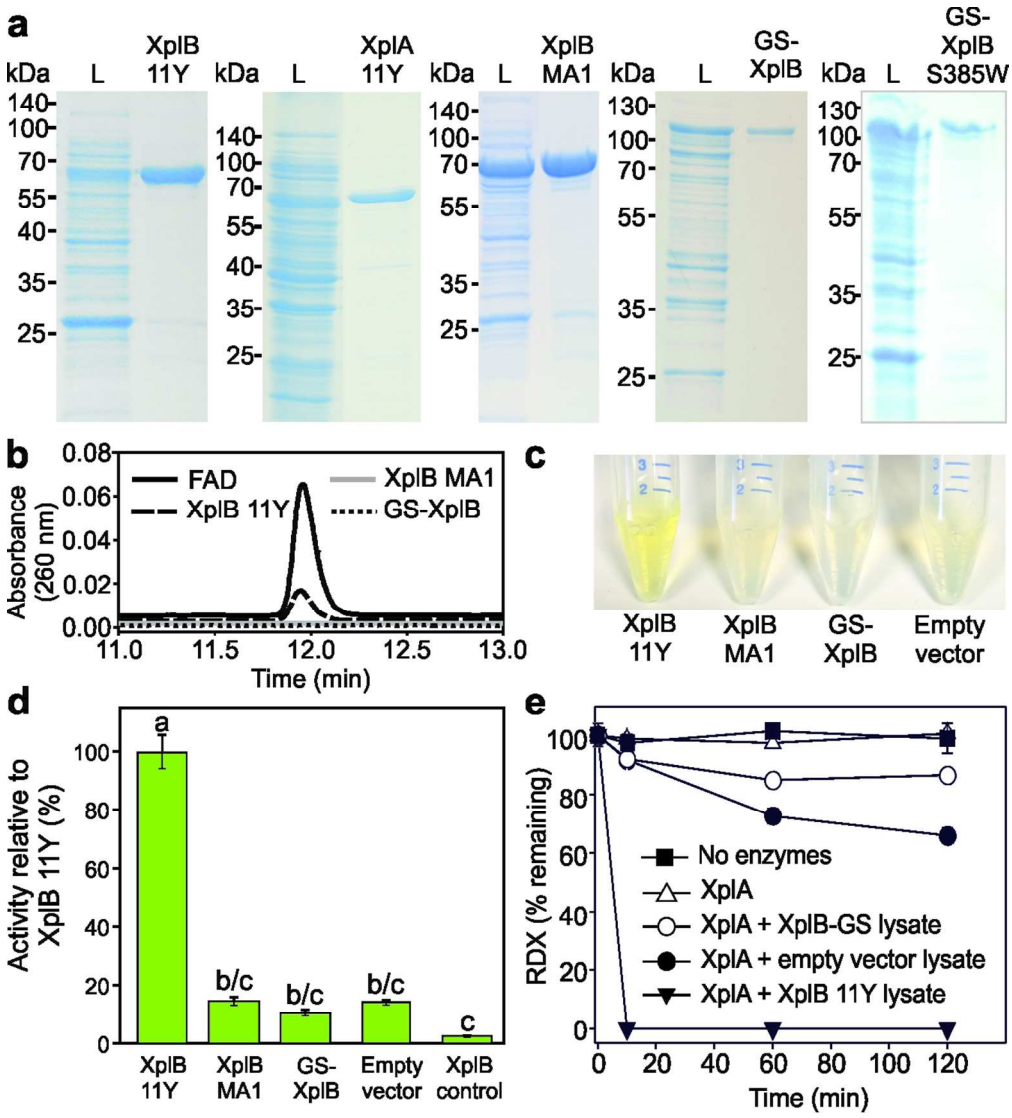

Figure 2. Purification and analysis of *R. rhodochrous* 11Y, *Microbacterium* sp. MA1 and *Gordonia* sp. KTR9 XplB‼ ┼ (a) SDS-PAGE analysis of lysates (L) and purified proteins from *E. coli* cells expressing XplA or XplB homologs. (b) Overlaid chromatograms showing FAD absorbance peaks from purified proteins. (c) Appearance of purified proteins. (d) Reductase activity in cell lysates expressing XplB homologs, measured using the Griess assay with purified XplA and RDX as substrate (n = 3 ± SD. Letters refer to significant differences; ANOVA, Tukey HSD). (e) RDX removal from *E. coli* cell lysates expressing *R. rhodochrous* 11Y XplB and *Gordonia* sp. KTR9 XplB-GS supplemented with purified XplA (n= 3 ± SD).

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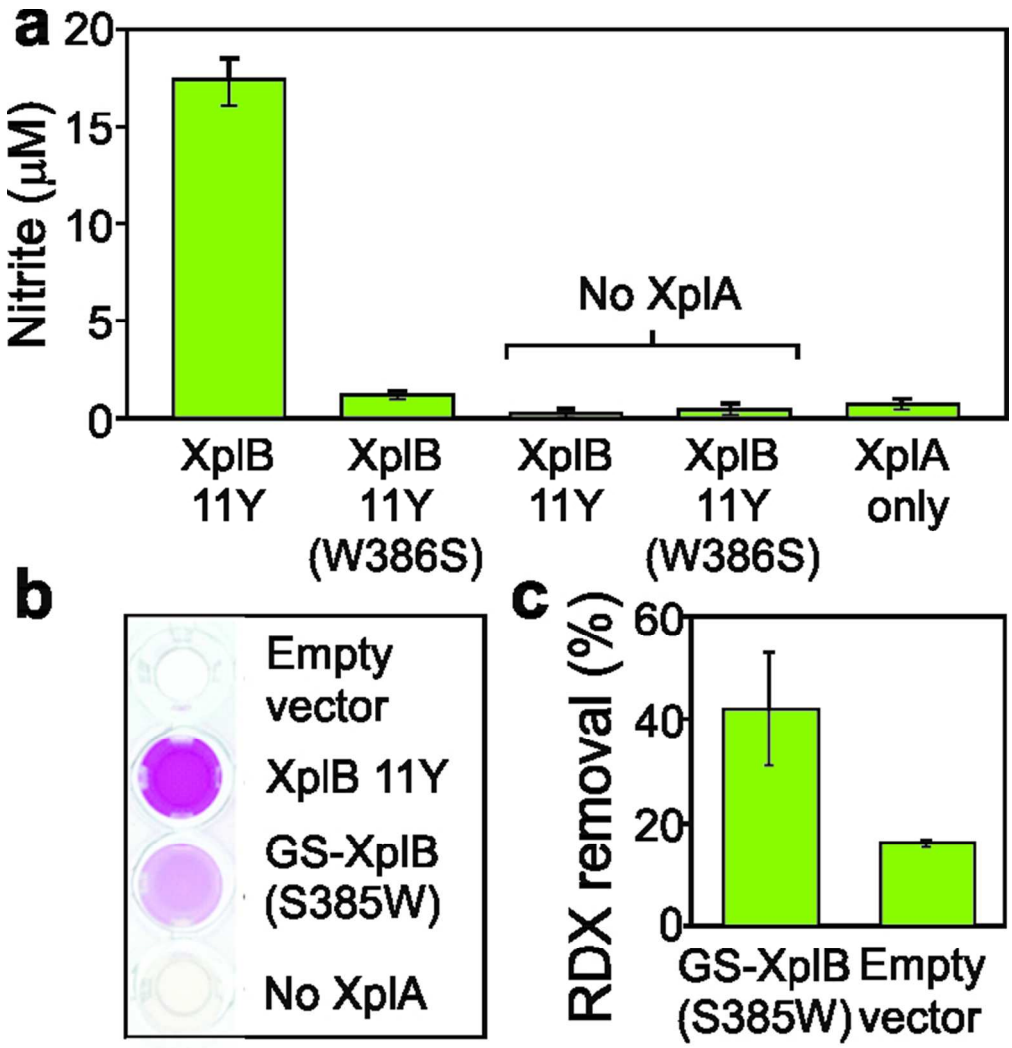

Figure 3 Activities of XplB proteins carrying reciprocal mutations from *R. rhodochrous* 11Y and *Gordonia* sp. KTR9.
(a) Nitrite release from *E. coli* cell lysates expressing *R. rhodochrous* 11Y XplB and *R. rhodochrous* 11Y XplB-(W386S) measured using the Griess assay, with purified XplA and RDX as substrate (n = 3 ± SD). (b) Nitrite release, observed using the Greiss assay, by *E. coli* cell lysates expressing *R. rhodochrous* 11Y XplB and *Gordonia* sp. KTR9 GS-XplB-(S385W), with purified XplA and RDX as substrate. (c) RDX removal by *E. coli* cell lysates expressing the *Gordonia* sp. KTR9 GS-XplB-(S385W) or empty vector (n = 3 ± SD).

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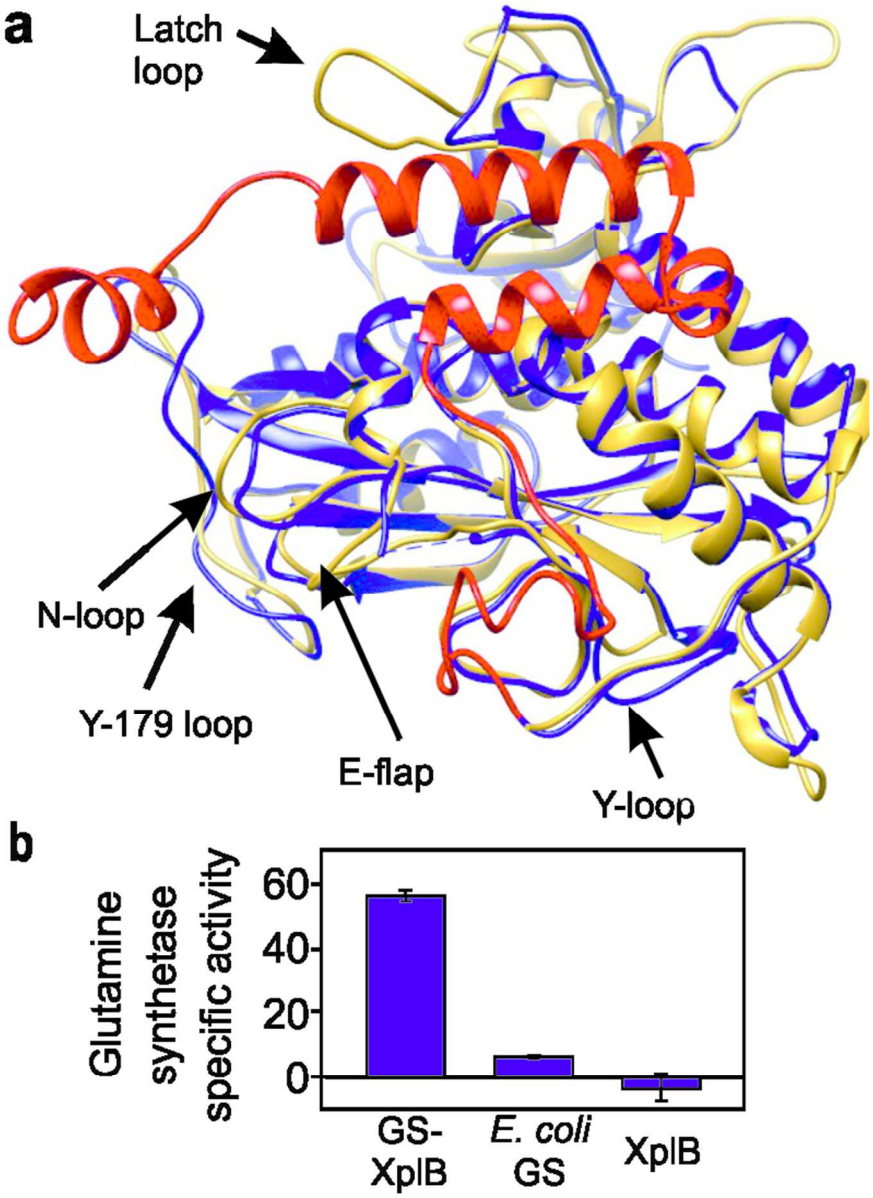

Figure 4 Characterization of the glutamine synthetase (GS) and XplB portions of Gordonia sp. KTR9 GS-XplB fusion protein.
(a) Model structure of GS from GS-XplB (blue) superimposed on the GS structure of B. subtilis (yellow). Sequence missing from GS-XplB (red). Root mean square deviation 1.14 ºA). (b) GS activity in E. coli cell lysates (n = 3 ± SD).

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